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Rac1, Rac2, RalA,[22] Rap2A, and Rap2B,[34] and several members of the Rab protein family.[11,23,40] The assay has also been used in conjunction with site-directed mutagenesis of Ras and Ras-related proteins to explore the importance of positioning of the target cysteine within the CaaX prenylation motif,[22] and to demonstrate that switching the terminal amino acid in the Ras sequence from S to L changes the protein from a FTase substrate to a GGTase I substrate.[25] We and others have begun to utilize the assay to define structural features beyond the carboxyl terminus that may be required for interaction of Rab proteins with GGTase II.[16,26,40] Because transcription and translation/prenylation of multiple mutant cDNAs can be completed more rapidly than analysis in intact cells, this method provides a convenient means to identify potentially informative mutants that are the best candidates for detailed study *in vivo*.

Acknowledgment

This work was supported in part by Grant CA34569 from the National Institutes of Health.

[40] M. Peter, P. Chavrier, E. A. Nigg, and M. Zerial, *J. Cell Sci.* **102,** 857 (1992).

[9] Prenylation and Carboxylmethylation of G-Protein γ Subunit

By YOSHITAKA FUKADA

Introduction

Heterotrimeric guanine nucleotide-binding proteins (G-proteins), composed of α, β, and γ subunits, mediate a variety of intracellular signal transductions by coupling activated membrane receptors with effector enzymes and channels. The α subunits bind GDP (inactive state) or GTP (active state) and play a central role in regulating activities of the effectors. The β and γ subunits are tightly associated with one another under physiological conditions, and the βγ complex is required for the GDP/GTP exchange reaction of the α subunit on activation of membrane receptors. To date, two different types of covalent lipid modifications have been identified in the α and γ subunits. Some members of the α subunits are modified

with fatty acids, myristate[1,2] (or structurally related fatty acids[3,4]) and/or palmitate.[5,6] On the other hand, all the known γ subunits are α-carboxylmethylated at the C-terminal cysteine residue,[7-11] to which C_{15} (farnesyl)[7,8] or C_{20} (geranylgeranyl)[9-11] polyisoprenoid is attached via a thioether bond. These modifications on the two subunits are implicated in the functions of signal-transducing G-proteins. The analysis of the structure and function of prenylation and methylation of G-protein γ subunits is described in this chapter.

Principle for Structural Analysis of γ Subunits

Complete and partial amino acid sequences of seven kinds of mammalian G-protein γ subunits (Fig. 1) have been determined mainly by the cDNA cloning technique. All the C-terminal sequences deduced from the cDNAs (γ_1, γ_2, γ_3, γ_5, and γ_7) contain the Cys-aa-X motif, which is a signal for the posttranslational modification (prenylation and carboxylmethylation) of the cysteine residue.[12-14] Some of the deduced sequences of the γ subunits have been confirmed by sequencing the purified polypeptides, but the C-terminal cysteine residue cannot be identified by usual Edman degradation, because the prenyl group is attached to a sulfur atom of the cysteine residue via a chemically stable thioether bond. On the other hand,

[1] A. M. Schultz, S.-C. Tsai, H.-F. Kung, S. Oroszlan, J. Moss, and M. Vaughan, *Biochem. Biophys. Res. Commun.* **146,** 1234 (1987).
[2] J. E. Buss, S. M. Mumby, P. J. Casey, A. G. Gilman, and B. M. Sefton, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7493 (1987).
[3] K. Kokame, Y. Fukada, T. Yoshizawa, T. Takao, and Y. Shimonishi, *Nature (London)* **359,** 749 (1992).
[4] T. A. Neubert, R. S. Johnson, J. B. Hurley, and K. A. Walsh, *J. Biol. Chem.* **267,** 18274 (1992).
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[6] M. Parenti, M. A. Vigano, C. M. Newman, G. Milligan, and A. I. Magee, *Biochem. J.* **291,** 349 (1993).
[7] Y. Fukada, T. Takao, H. Ohguro, T. Yoshizawa, T. Akino, and Y. Shimonishi, *Nature (London)* **346,** 658 (1990).
[8] R. K. Lai, D. Pérez-Sala, F. J. Cañada, and R. R. Rando, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 7673 (1990).
[9] H. K. Yamane, C. C. Farnsworth, H. Xie, W. Howald, B. K.-K. Fung, S. Clarke, M. H. Gelb, and J. A. Glomset, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 5868 (1990).
[10] S. M. Mumby, P. J. Casey, A. G. Gilman, S. Gutowski, and P. C. Sternweis, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 5873 (1990).
[11] R. Morishita, Y. Fukada, K. Kokame, T. Yoshizawa, K. Masuda, M. Niwa, K. Kato, and T. Asano, *Eur. J. Biochem.* **210,** 1061 (1992).
[12] S. Clarke, *Annu. Rev. Biochem.* **61,** 355 (1992).
[13] Y. Reiss, this volume [3].
[14] J. F. Mooman, F. L. Zhang, and P. J. Casey, this volume [2].

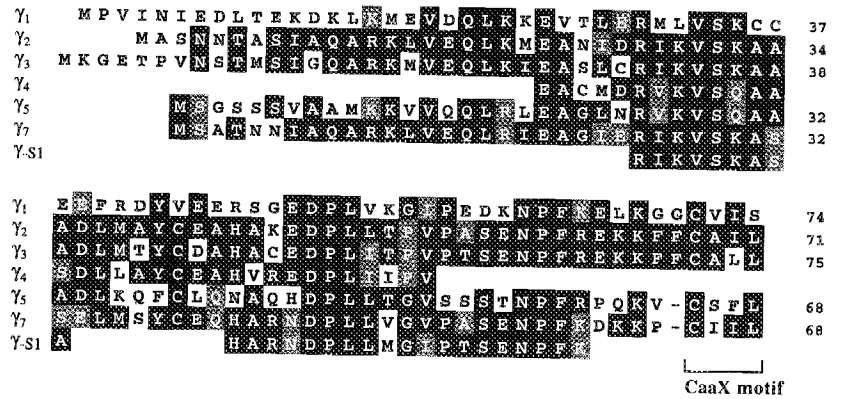

FIG. 1. Alignment of amino acid sequences of mammalian G-protein γ subunits. Only partial sequences of γ_4 and γ_{-S1} have been determined. The amino acids conserved in two or more isoforms are shown by white letters on a black background. When two kinds of amino acid conservations are observed in a single position, another amino acid is shown by white letters on a shaded background. Gaps (–) are introduced for optimal alignment.

the methyl ester is susceptible to hydrolysis at higher pH, and therefore the prenylation and methylation of a peptide have to be verified under carefully controlled conditions.

Raney nickel has been widely used to cleave the thioether bond, and the structure of the released isoprenoid is then identified by gas chromatography-coupled mass spectrometry.[8,9,11] Methyl iodide is also used to release isoprenoids from peptides,[8,10] and these procedures have been summarized.[15] Alternatively, nascent polypeptides are modified with an isotopically labeled precursor of farnesyl, geranylgeranyl, and methyl groups, and then the structures of the radioactive moieties attached to the peptides are determined.[16] Another approach, which is summarized in this chapter, employs enzymatic proteolysis of modified proteins to isolate a short prenylated peptide, whose structure is then analyzed with the aid of mass spectrometry. This method allows simple determination of the prenylated site (amino acid residue) within the polypeptide and enables one to estimate the stoichiometry of the modification. The latter is important for evaluating effects of the modifications on G-protein functions. In the case of transducin, a G-protein involved in the photon-signal transduction of vertebrate photo-

[15] C. C. Farnsworth, P. J. Casey, W. N. Howald, J. A. Glomset, and M. H. Gelb, *Methods (San Diego)* **1,** 231 (1990).

[16] H. Xie, H. K. Yamane, R. C. Stephenson, O. C. Ong, B. K.-K. Fung, and S. Clarke, *Methods (San Diego)* **1,** 276 (1990).

receptors, one can isolate and quantify three subspecies of the γ subunit, the C termini of which are processed differently.

Because every γ subunit of G-proteins is tightly complexed with β subunit (β_1–β_4), the $\beta\gamma$ complex must be denatured for isolation of the γ subunit to be analyzed. Initially, gel-filtration column chromatography at neutral pH in the presence of 6 *M* guanidine hydrochloride was used to isolate the γ subunit of transducin (T$\alpha\beta\gamma$).[17] This method, however, gives a low yield of isolated Tγ, probably because of incomplete dissociation of the β and γ subunits and broad elution of the dissociated β subunit. The γ subunit can be purified more conveniently and completely by reversed-phase high-performance liquid chromatography (HPLC) under acidic conditions in the presence of 0.05–0.1% (v/v) trifluoroacetic acid.[9,11,18] It seems likely that β and γ subunits associate with one another through ionic interactions which could be weakened by lowering the pH. In addition, β subunit dissociated from the γ subunit becomes highly hydrophobic and hence adsorbs irreversibly to the column beads. This behavior of the β subunit increases the purity of the dissociated γ subunit.

Purification of γ Subunits of Transducin and Other G-Proteins

The $\beta\gamma$ complex (T$\beta\gamma$) of bovine transducin is composed of two forms, tentatively termed T$\beta\gamma$-1 and T$\beta\gamma$-2, each of which has its own γ subunit, Tγ-1 and Tγ-2, showing different electrophoretic mobilities on urea/sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[17] (apparent molecular weights: Tγ-1, 8000; Tγ-2, 6000). T$\beta\gamma$-1 and T$\beta\gamma$-2 can be isolated by chromatography on a Mono Q HR 5/5 column (Pharmacia, Piscataway, NJ) under nondenaturing conditions.[17] To analyze the structural difference between the γ subunits, T$\beta\gamma$-1 and T$\beta\gamma$-2 are separately injected into a Cosmosil $5C_{18}$-P300 reversed-phase column (4.6 × 150 mm; Nacalai Tesque, Kyoto, Japan) and eluted with a linear gradient of acetonitrile (5–75%, 1%/min) in 0.05% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min. As shown in Fig. 2, Tγ-1 is eluted in a single peak at a retention time of 51 min. On the other hand, injection of T$\beta\gamma$-2 results in separation of Tγ-2 into two peaks, designated Tγ-2a and Tγ-2b, at retention times of 56 and 57 min, respectively. Their apparent molecular weight (6000) estimated by urea/SDS–PAGE (Fig. 2, inset, lanes 2a and 2b) coincides with that of Tγ-2. The N-terminal amino acid sequences of Tγ-1, Tγ-2a, and Tγ-2b thus isolated are identical with that deduced from the cDNA (Fig.

[17] Y. Fukada, H. Ohguro, T. Saito, T. Yoshizawa, and T. Akino, *J. Biol. Chem.* **264,** 5937 (1989).
[18] H. Ohguro, Y. Fukada, T. Takao, Y. Shimonishi, T. Yoshizawa, and T. Akino, *EMBO J.* **10,** 3669 (1991).

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s is tightly complexed with β subunit tured for isolation of the γ subunit column chromatography at neutral drochloride was used to isolate the method, however, gives a low yield complete dissociation of the β and ssociated β subunit. The γ subunit completely by reversed-phase high- PLC) under acidic conditions in the etic acid.[9,11,18] It seems likely that β er through ionic interactions which . In addition, β subunit dissociated phobic and hence adsorbs irrevers- ior of the β subunit increases the

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nd T. Akino, *J. Biol. Chem.* **264,** 5937 (1989).
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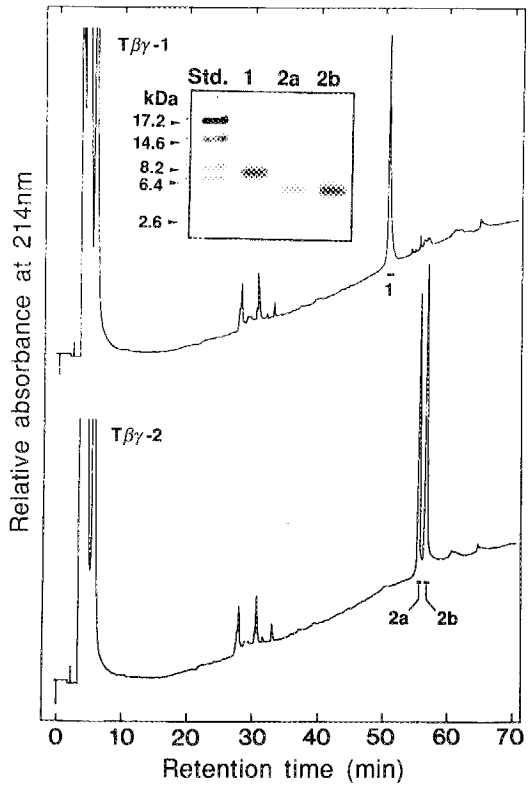


FIG. 2. Isolation of bovine transducin γ subunit using reversed-phase HPLC. Purified Tβγ-1 (top trace) or Tβγ-2 (bottom) was loaded onto a Cosmosil $5C_{18}$-P300 reversed-phase column equipped with an HPLC system (Model 600E; Waters, Milford, MA). The γ subunits were eluted under the conditions described in the text. The absorbance at 214 nm of the eluate was continuously monitored. Three fractions indicated by the horizontal bars (Tγ-1, Tγ-2a, and Tγ-2b) were collected, lyophilized, and subjected to urea/SDS–PAGE (inset). Lane Std contained molecular weight standard proteins (Pharmacia). [Reprinted with permission of Oxford University Press, from H. Ohguro, Y. Fukada, T. Takao, Y. Shimonishi, T. Yoshizawa, and T. Akino, *EMBO J.* **10,** 3669 (1991).]

1, γ_1) except that the initial methionine residue in each subspecies is removed to have a proline at the N terminus. Approximately 90% of the injected γ subunit is reproducibly recovered from the column based on quantification by amino acid analysis. Owing to the high recovery, one can estimate the relative content of Tγ subspecies (i.e., stoichiometry of modifications) by comparing areas (or, more precisely, protein concentrations) of the peaks in the reversed-phase HPLC analysis.

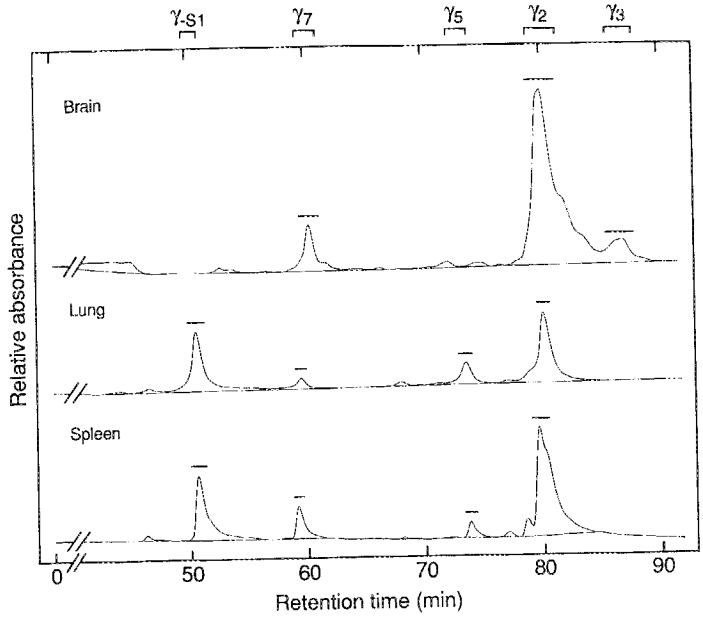
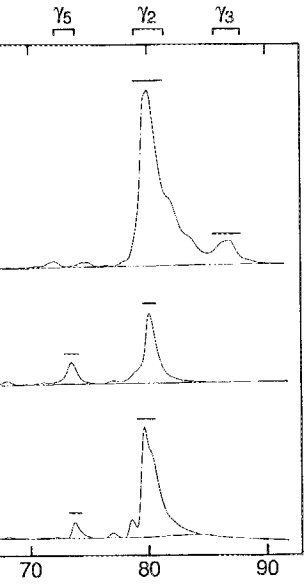


FIG. 3. Isolation of bovine G-protein γ subunits using reversed-phase HPLC. The $\beta\gamma$ complexes purified from bovine brain (top trace), lung (middle), and spleen (bottom) were loaded onto a Cosmosil 5PE reversed-phase column. The γ subunits were eluted under the conditions described in the text. The absorbance at 214 nm of the eluate was continuously monitored. Each peak fraction (indicated with bars) was shown to contain a distinct isoform of γ subunits [modified from R. Morishita, Y. Fukada, K. Kokame, T. Yoshizawa, K. Masuda, M. Niwa, K. Kato, and T. Asano, *Eur. J. Biochem.* **210,** 1061 (1992)]. Some of the γ subunits were assigned in another source [R. Morishita, K. Masuda, M. Niwa, K. Kato, and T. Asano, *Biochem. Biophys. Res. Commun.* **194,** 1221 (1993)].

By employing a similar procedure, one can isolate the other G-protein γ subunits from $\beta\gamma$ complexes purified from various tissues.[9,11] Unlike the transducin $\beta\gamma$ complex ($\beta_1\gamma_1$) of bovine retinal rod cells, G-protein $\beta\gamma$ complexes purified from bovine brain, lung, and spleen contain distinct sets of γ subunits with diverse amino acid sequences.[11] Some of the $\beta\gamma$ complexes ($\beta_1\gamma_2$, $\beta_1\gamma_3$, $\beta_1\gamma_7$, $\beta_1\gamma_{-S1}$) have been purified from bovine brain and spleen under nondenaturing conditions, and their functions have been compared with one another.[19] For structural analysis of the γ subunits, however, a mixture of $\beta\gamma$ complexes isolated from a tissue can be directly subjected to the reversed-phase HPLC step, which allows the isolation of each γ subunit as shown in Fig. 3. The experiment employs 1–2 mg $\beta\gamma$ complexes

[19] T. Asano, R. Morishita, T. Matsuda, Y. Fukada, T. Yoshizawa, and K. Kato, *J. Biol Chem.* **268,** 20512 (1993).



nits using reversed-phase HPLC. The βγ lung (middle), and spleen (bottom) were nn. The γ subunits were eluted under the at 214 nm of the eluate was continuously s) was shown to contain a distinct isoform da, K. Kokame, T. Yoshizawa, K. Masuda, **210,** 1061 (1992)]. Some of the γ subunits Masuda, M. Niwa, K. Kato, and T. Asano, 3)].

ne can isolate the other G-protein rom various tissues.[9,11] Unlike the e retinal rod cells, G-protein βγ ng, and spleen contain distinct sets ences.[11] Some of the βγ complexes fied from bovine brain and spleen eir functions have been compared ysis of the γ subunits, however, a a tissue can be directly subjected ch allows the isolation of each γ ent employs 1–2 mg βγ complexes

T. Yoshizawa, and K. Kato, *J. Biol Chem.*

purified from bovine brain, lung, and spleen.[11] The βγ complexes are injected into a reversed-phase Cosmosil 5PE (phenylethyl) column (8 × 250 mm; Nacalai Tesque) preequilibrated with solvent A (10% acetonitrile/90% water in 0.1% trifluoroacetic acid, in v/v). The γ subunits are eluted with an increasing concentration of solvent B (80% acetonitrile/10% 2-propanol/10% water in 0.1% trifluoroacetic acid, in v/v) at a flow rate of 2 ml/min. The concentration of solvent B is increased from 0 to 40% over 25 min, then to 65% over 100 min.

Isolation and Analysis of Proteolytic Fragments of Transducin γ Subunit

Primary structures (including modifications) of the γ subunits isolated by reversed-phase HPLC (Figs. 2, 3) are investigated by separating the proteolytic fragments. As a typical example, the structural analysis of the subspecies of transducin γ subunit is summarized.[7,18] Other G-protein γ subunits can be analyzed in a similar way.[11]

The three subspecies of Tγ (Tγ-1, Tγ-2a, and Tγ-2b; 1–2 nmol each) isolated by reversed-phase HPLC (Fig. 2) are lyophilized and incubated with 1 μg of *Staphylococcus aureus* protease V8 (Miles Laboratories, Naperville, IL) in 50 μl of 12.5 m*M* NH_4HCO_3 (pH 7.8) at 37° for 6 hr for complete digestion. The digest is then loaded onto the Cosmosil $5C_{18}$-P300 reversed-phase column, and the proteolytic fragments are separated from one another by elution with a linear gradient of acetonitrile as shown in Fig. 4. Elution positions of fragments 1–9 (designated in Fig. 4) derived from the three Tγ subspecies are identical with one another, whereas fragments 10 and 11 are characteristic of Tγ-2a and Tγ-2b, respectively.

Sequence analyses and fast atom bombardment (FAB) mass spectra of fragments 1–9 show no difference in structure from Pro-1 through Glu-65 among the subspecies (fragments: 1, Val^{18}–Glu^{24}; 2, Val^{25}–Glu^{28}; 3, Lys^{11}–Glu^{17}; 4, Asp^{59}–Glu^{65}; 5, Phe^{39}–Glu^{45}; 6, Arg^{29}–Glu^{38}; 7, Arg^{46}–Glu^{58}; 8, Arg^{46}–Glu^{65}; 9, Pro^{1}–Glu^{10}). The FAB mass spectrum of fragment 6 indicates that the two neighboring cysteine residues (Cys-35, Cys-36) form a disulfide bond in every subspecies. The C-terminal fragments 10 and 11 have an identical sequence Leu^{66}–Gly^{69}, but the presence of a cysteine residue in both fragments is confirmed by amino acid analyses, indicating the C-terminal sequence of Tγ-2a (fragment 10) and Tγ-2b (fragment 11) to be Leu-Lys-Gly-Gly-Cys, which is deduced from the cDNA (Fig. 1).

On the other hand, the C-terminal proteolytic fragment of Tγ-1 flows through the reversed-phase column (Fig. 4, top trace). To determine precisely the C-terminal structure, a longer C-terminal fragment of Tγ-1 is obtained by incubating purified Tγ-1 (1 nmol) at 37° for 48 hr in 50 μl of

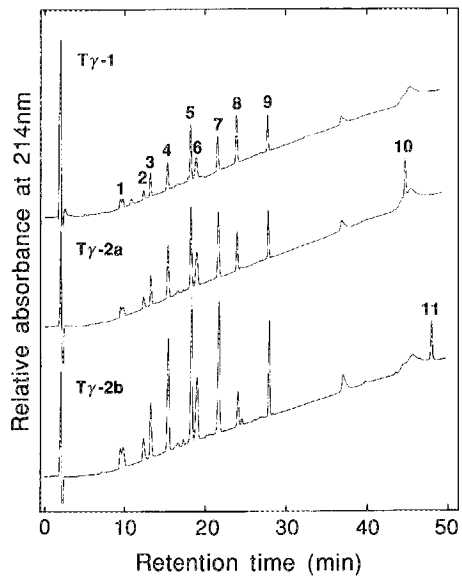
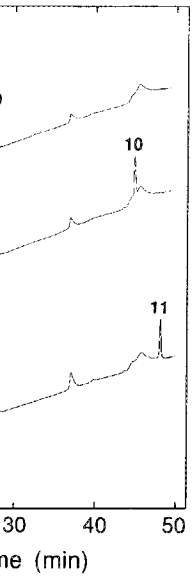


FIG. 4. Elution profile of proteolytic fragments derived from Tγ subspecies. Proteolytic fragments of Tγ-1 (top trace), Tγ-2a (middle), or Tγ-2b (bottom) digested with *S. aureus* protease V8 were separated from one another by the Cosmosil $5C_{18}$-P300 reversed-phase column as described in the legend to Fig. 2. Broad peaks at retention times of 37 and 46 min were attributed to the V8 protease and some impurity in the solvent, respectively. [Reprinted, with permission of Oxford University Press, from H. Ohguro, Y. Fukada, T. Takao, Y. Shimonishi, T. Yoshizawa, and T. Akino, *EMBO J.* **10,** 3669 (1991).]

70% formic acid to cleave between Asp-50 and Pro-51.[20] The C-terminal fragment can be isolated by reversed-phase HPLC on the Cosmosil $5C_{18}$-P300 column under the same conditions as described in Fig. 2. The amino acid sequence of the fragment thus isolated is identical with the corresponding part of Tγ-2 (Pro^{51}–Gly^{69}), and the amount of the last glycine residue (Gly-69) is extremely low in the sequence analysis. This is consistent with the following mass spectrometry data indicating that part of Tγ-1 lacks Gly-69. The FAB mass spectrum of the C-terminal fragment displays two signals at *m/z* 2008.9 and 2066.0, which coincide with the calculated masses of Pro^{51}–Gly^{68} (2009.1) and Pro^{51}–Gly^{69} (2066.2), respectively. It is concluded that Tγ-1 is a mixture of peptides, Pro^{1}–Gly^{68} and Pro^{1}–Gly^{69}, both lacking the C-terminal cysteine residue (Fig. 5).

[20] D. Piszkiewicz, M. Landon, and E. L. Smith, *Biochem. Biophys. Res. Commun.* **40,** 1173 (1970).



s derived from Tγ subspecies. Proteolytic
r Tγ-2b (bottom) digested with *S. aureus*
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-50 and Pro-51.[20] The C-terminal
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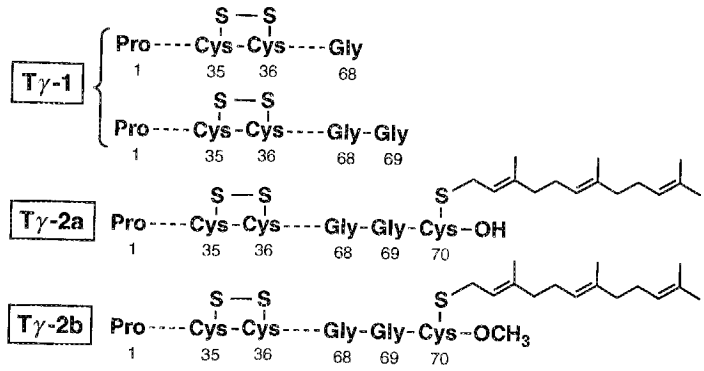

FIG. 5. Structures of three subspecies of bovine Tγ.

Identification of Farnesylation and Carboxylmethylation of Tγ-2

The remarkable difference in retention time on the reversed-phase column (Fig. 4) among the C-terminal peptides of Tγ-1 (Leu^{66}–Gly^{68} and Leu^{66}–Gly^{69}; flow-through fraction), Tγ-2a (Leu^{66}–Cys^{70}; fragment 10), and Tγ-2b (Leu^{66}–Cys^{70}; fragment 11) suggests that the cysteine residue at the C terminus of Tγ-2 is heterogeneously modified with hydrophobic group(s). One can assume two substituents X_1 attached to the sulfur atom and X_2 to the carboxyl group of the C-terminal cysteine residue. The elemental compositions of X_1 and X_2 are determined by accurate FAB mass spectra of fragments 10 and 11 as follows.

Lyophilized fragment 10 or 11 is dissolved in 50% aqueous acetonitrile, and the aliquot (~0.1 nmol) is mixed with dithiothreitol/dithioerythritol (5:1, w/w) on the target. Accurate FAB mass spectra are measured by a double-focusing mass spectrometer (Jeol JMS-HX100, Tokyo, Japan) equipped with a FAB ion source.[21] A parent ion peak is observed at *m/z* 681.438 for fragment 10 or at *m/z* 695.453 for fragment 11. The accurate mass of the substituents (sum of X_1 and X_2) is calculated to be 222.199 (fragment 10) or 236.214 (fragment 11), giving the unique elemental compositions $C_{15}H_{26}O$ (theoretical mass 222.200) and $C_{16}H_{28}O$ (theoretical mass 236.214) for fragment 10 and 11, respectively, the latter having an additional CH_2. This strongly suggests that fragment 11 is the α-carboxylmethylated form of fragment 10. Then it is tested whether fragment 11 is converted to fragment 10 on alkaline treatment, because the methyl ester bond is a base-labile linkage. When fragment 11 (0.3 nmol) is incubated at 4° for 2

[21] M. Kikuchi, Y. Taniyama, S. Kanaya, T. Takao, and Y. Shimonishi, *Eur. J. Biochem.* **187,** 315 (1990).

hr in 50 μl of 60 m*M* NaOH in 40% (v/v) methanol, the retention time of saponified fragment 11 in the reversed-phase column shifts to that of fragment 10, indicating that X_2 in fragments 10 and 11 are OH and OCH_3, respectively. The residual elemental composition (X_1) in both fragments coincides with that of a farnesyl group ($C_{15}H_{25}$).

Finally, the structure of the modifying group should be verified by identification of fragment 10 with chemically synthesized peptide. The farnesylated peptide [Leu^{66}–Cys^{70} (*S-trans,trans*-farnesyl)] can be synthesized chemically from a pentapeptide, Leu-Lys-Gly-Gly-Cys, and *trans,trans*-farnesyl bromide.[22] The farnesylated peptide thus synthesized is subjected to the reversed-phase HPLC step to show that the retention time is identical with that of fragment 10. The identity is confirmed by the fact that an equimolar mixture of fragment 10 and the synthetic peptide is eluted in a single peak from the Cosmosil $5C_{18}$-P300 column in two different solvent systems (a linear gradient of acetonitrile in 0.05% v/v aqueous trifluoroacetic acid and in 10 m*M* ammonium acetate at pH 5.7), and that they both show exactly the same mass spectrum.[7] It is concluded that Tγ-2 is composed of 70 amino acids and has an S-farnesylated cysteine residue at the C terminus (Tγ-2a), a part of which is additionally methyl-esterified at the α-carboxyl group (Tγ-2b). The overall structures (Fig. 5), including the absence of other modifications, are confirmed by ion-spray mass spectrometry, which shows that the molecular weights of intact Tγ-2a (8315.7) and Tγ-2b (8330.2) are in good agreement with the calculated values of nonmethylated (8315.7) and α-carboxylmethylated forms (8329.7) of Tγ-2 [Pro^1–Cys^{70}(*S*-farnesyl)].[18] It seems likely that the electrophoretic mobility of the γ subunit is increased by the prenylation but not by the methylation (Fig. 2, inset).

Functional Roles of Farnesylation and Methylation of Tγ

The γ subunit of transducin (γ_1) is farnesylated,[7,8] whereas the other G-protein γ subunits (γ_2,[9–11] γ_3,[11] γ_7,[9,10] γ_{-s1}[11,23]) are geranylgeranylated at the C-terminal cysteine residue, the α-carboxyl group of which is methylated in every case. In the next stage of investigation, it is necessary to define the roles of prenylation and methylation in G-protein function, and to examine whether the difference in chain length between the prenyl groups might have a biological significance. Comparison of the function between Tβγ (farnesylated) and other G-protein βγ subunits (geranylgera-

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'v) methanol, the retention time of phase column shifts to that of frag- nts 10 and 11 are OH and OCH_3, mposition (X_1) in both fragments ($C_{15}H_{25}$).

fying group should be verified by cally synthesized peptide. The farn- *trans*-farnesyl)] can be synthesized -Lys-Gly-Gly-Cys, and *trans,trans*- eptide thus synthesized is subjected w that the retention time is identical is confirmed by the fact that an the synthetic peptide is eluted in a 00 column in two different solvent in 0.05% v/v aqueous trifluoroacetic at pH 5.7), and that they both show oncluded that Tγ-2 is composed of d cysteine residue at the C terminus methyl-esterified at the α-carboxyl (Fig. 5), including the absence of on-spray mass spectrometry, which intact Tγ-2a (8315.7) and Tγ-2b calculated values of nonmethylated forms (8329.7) of Tγ-2 [Pro^1– the electrophoretic mobility of the on but not by the methylation (Fig.

ad Methylation of Tγ

farnesylated,[7,8] whereas the other [10] γ_{-S1}[11,23]) are geranylgeranylated -carboxyl group of which is methyl- of investigation, it is necessary to hylation in G-protein function, and chain length between the prenyl cance. Comparison of the function G-protein βγ subunits (geranylgera-

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nylated)[19,24] could provide a clue to the latter issue. In general, it has been proposed that covalent lipid modifications play a predominant role in the targeting of modified proteins to cellular membranes. For instance, geranylgeranylation of the γ subunit of brain G protein leads to a tight membrane association of the βγ complex.[25] Accompanying methylation could play a supplementary role in membrane association, as the hydrophobicity of the prenylated protein is increased by the methylation, which blocks the carboxylate anion.

In the case of transducin, however, the C-terminal cysteine residue in Tγ has been shown to provide a specific Tα–Tβγ interaction, which is indispensable for the function of transducin. The original finding was that Tβγ-1 showed extremely low activity (~3% to that of Tβγ-2) in enhancing the GDP/GTP exchange reaction on Tα catalyzed by light-activated rhodopsin (see also Fig. 6).[17] Later, this difference was ascribed to the reduced affinity of Tβγ-1 for Tα-GDP,[26] and eventually a farnesylated and partly methylated cysteine residue was identified in Tβγ-2 but not in Tβγ-1 (Fig. 5).[7] Thus the functional α–βγ interaction of transducin was shown to absolutely require the modified cysteine residue in Tγ, though the contribution of the methylation remained to be examined. It should be noted that the α–βγ interaction of other G-proteins also requires prenylation or methylation (or both) of the γ subunit.[27]

The carboxylmethylated (Tβγ-2b) and nonmethylated forms (Tβγ-2a) of farnesylated Tβγ have been separated from one another by Superdex 75 column (Pharmacia) chromatography.[28] The isolation of Tβγ-2a and Tβγ-2b under nondenaturing conditions enables one to define the role of the methylation of Tγ. Because the GDP/GTP exchange reaction on Tα requires both Tβγ and a photobleaching intermediate of rhodopsin (metarhodopsin II), the activity of Tβγ can be assessed by measuring the rate of guanosine 5′-*O*-3-(thio)triphosphate (GTPγS, a nonhydrolyzable analog of GTP) binding to a fixed amount of Tα in the presence of light-activated rhodopsin reconstituted in phosphatidylcholine.[17,28] As shown in Fig. 6A,

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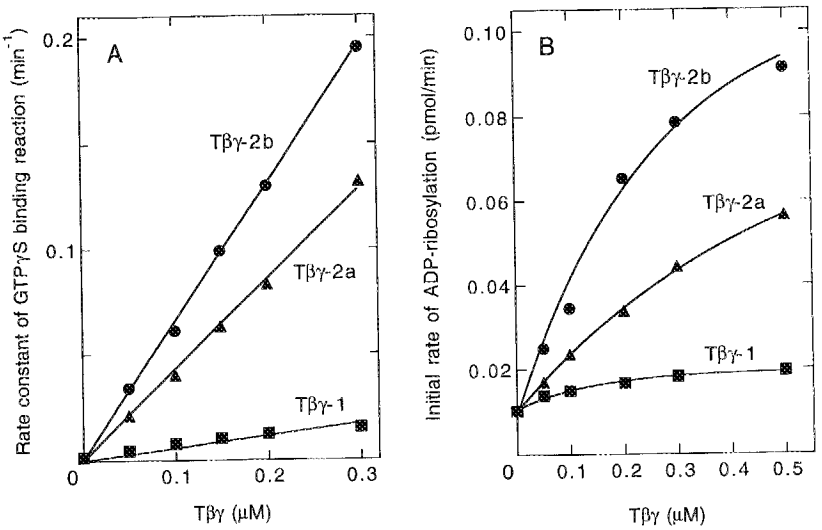
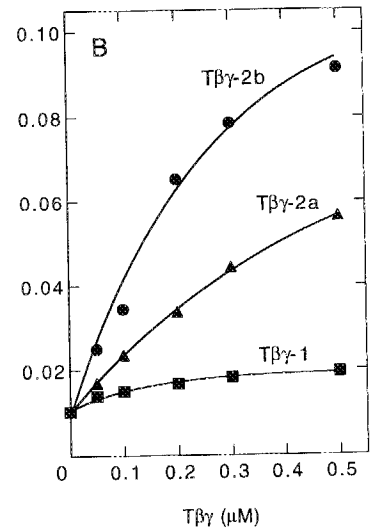


FIG. 6. Effects of modifications of Tγ on Tβγ activities stimulating (A) GTPγS binding to Tα catalyzed by light-activated rhodopsin and (B) ADP-ribosylation of Tα catalyzed by pertussis toxin. (A) Time courses of the GTPγS binding to Tα were measured at 4° in reaction mixtures (150 μl) composed of various concentrations of Tβγ, 0.8 μ*M* Tα, 30 n*M* metarhodopsin II in liposomes, 2.4 mg/ml ovalbumin, 0.002% Lubrol PX, and 10 μ*M* [^{35}S]GTPγS (~3 Ci/mmol) in 10 m*M* MOPS–NaOH buffer (pH 7.5) containing 100 m*M* NaCl, 2 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 0.1 m*M* phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, and 50 kallikrein inhibitor units/ml aprotinin. Reactions were started by the addition of [^{35}S]GTPγS and terminated at selected time intervals by diluting 10-μl aliquots of the reaction mixture into 0.18 ml of 100 m*M* Tris-HCl buffer (pH 7.5) supplemented with 1 m*M* $MgCl_2$ and 2 m*M* GTP. The [^{35}S]GTPγS bound to Tα was isolated from free [^{35}S]GTPγS by filtering the samples over 0.45 μm cellulose membranes (type HATF; Millipore, Bedford, MA) fitted with a MultiScreen Assay System (Millipore). Immediately after the filtration, the membranes were washed 4 times with 0.2 ml of the Tris buffer, dried, and then counted. The data points were best-fitted to a single exponential equation, $B(t) = B_m[1 - \exp(kt)]$, where $B(t)$ is the amount of GTPγS bound to Tα at time t, B_m is the maximum binding at infinite time, and k is the rate constant. The rate constants were plotted against the concentrations of Tβγ-1 (squares), Tβγ-2a (triangles), and Tβγ-2b (circles). (B) Time courses of the ADP-ribosylation catalyzed by pertussis toxin were measured in reaction mixtures composed of various concentrations of Tβγ, 10 μg/ml preactivated pertussis toxin (Kaken-Seiyaku Co., Tokyo, Japan), 10 μ*M* [α-^{32}P]NAD (1.0 Ci/mmol), 0.5 μ*M* Tα, 1.8 mg/ml ovalbumin, and 0.005% Lubrol PX. After incubation at 30°, reactions were terminated at selected time intervals by diluting 10-μl aliquots of the reaction mixture into 0.18 ml of 100 m*M* Tris-HCl buffer (pH 7.5) supplemented with 1 m*M* $MgCl_2$, 1 m*M* NAD, and 13.2% (w/v) trichloroacetic acid. The [^{32}P]ADP-ribosylated Tα was isolated from free [α-^{32}P]NAD according to the filtration method described above. The initial rates of the reactions were calculated from linear fitting of the data and plotted against the concentrations of Tβγ-1 (squares), Tβγ-2a (triangles), and Tβγ-2b (circles).



activities stimulating (A) GTPγS binding (B) ADP-ribosylation of Tα catalyzed by S binding to Tα were measured at 4° in concentrations of Tβγ, 0.8 μ*M* Tα, 30 n*M* lbumin, 0.002% Lubrol PX, and 10 μ*M* aOH buffer (pH 7.5) containing 100 m*M* *M* phenylmethylsulfonyl fluoride, 4 μg/ml tinin. Reactions were started by the addition intervals by diluting 10-μl aliquots of the l buffer (pH 7.5) supplemented with 1 m*M* o Tα was isolated from free [^{35}S]GTPγS by mbranes (type HATF; Millipore, Bedford, lipore). Immediately after the filtration, the ie Tris buffer, dried, and then counted. The al equation, $B(t) = B_m[1 - \exp(kt)]$, where ne t, B_m is the maximum binding at infinite nts were plotted against the concentrations 2b (circles). (B) Time courses of the ADP- easured in reaction mixtures composed of ivated pertussis toxin (Kaken-Seiyaku Co., ol), 0.5 μ*M* Tα, 1.8 mg/ml ovalbumin, and ns were terminated at selected time intervals e into 0.18 ml of 100 m*M* Tris-HCl buffer NAD, and 13.2% (w/v) trichloroacetic acid. free [α-^{32}P]NAD according to the filtration reactions were calculated from linear fitting s of Tβγ-1 (squares), Tβγ-2a (triangles), and

only slight stimulation of the reaction is observed by the addition of increasing amounts of Tβγ-1, indicating a crucial role of the modified cysteine residue in the function of transducin. On the other hand, the activity of Tβγ-2a (nonmethylated form) is enhanced about 50% by methylation of Tγ (Fig. 6A, compare Tβγ-2b with Tβγ-2a). Clearly, both farnesylation and methylation of Tγ support the highest turnover rate of GDP/GTP exchange for transducin.

Similar effects of the double modifications are observed in the ADP-ribosylation reaction of Tα catalyzed by pertussis toxin in the absence of metarhodopsin II and membranes (Fig. 6B). Because pertussis toxin ADP-ribosylates Tα-GDP complexed with Tβγ, quantitative analysis of the reaction rate is one of the most sensitive methods to detect physical coupling between Tα and Tβγ in solution.[29] In fact, the initial rate of the reaction is enhanced progressively with increasing concentrations of farnesylated Tβγ (Tβγ-2a and Tβγ-2b) as shown in Fig. 6B. At a fixed concentration of Tβγ, the reaction rate is increased about 2-fold by the methylation (compare Tβγ-2b with Tβγ-2a), indicating that the methylation noticeably stabilizes the trimeric (Tα–Tβγ) complex in solution. This effect should be primarily responsible for the acceleration of the GTPγS binding reaction, which requires interaction between trimeric transducin and metarhodopsin II in membranes. Lack of stimulation of the ADP-ribosylation reaction by Tβγ-1 lacking the modified cysteine residue supports the idea that the double modifications provide a specific protein–protein interaction between Tα-GDP and Tβγ. These observations suggest that Tα-GDP might have an unidentified hydrophobic cleft accommodating the farnesyl and methyl moieties which enable Tβγ to associate efficiently with Tα-GDP. The modification seems to act as a "molecular antenna" recognizing the specific target. In fact, a small synthetic peptide corresponding to the C-terminal part of Tγ competitively inhibited the α-βγ interaction only when the peptide was prenylated.[30] Interestingly, the geranylgeranylated peptide was a more potent inhibitor than the farnesylated one, suggesting that the alkyl chain length of the prenyl group would regulate the strength of the α-βγ interaction of G-proteins.[30] The exact nature of the interaction remains to be elucidated.

On the other hand, the modifications seem to have another role after activation of transducin, as Tα and Tβγ dissociate from one another on GDP/GTP exchange reaction on Tα. In the dissociated state, the double

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modifications on Tγ are required for membrane association of released Tβγ.[28] Such an effect of prenylation has been well documented in many cases, but it should be emphasized that the nonmethylated form of farnesylated Tβγ (Tβγ-2a) shows an extremely low affinity for the dark-adapted and irradiated photoreceptor membranes, as compared with doubly modified Tβγ (Tβγ-2b).[28] The effect of methylation on membrane association of prenylated protein has been less stressed, but in the case of transducin, farnesylation of Tγ by itself is not sufficient to anchor Tβγ at the membrane surface. This is consistent with the finding that neither farnesylation nor geranylgeranylation is sufficient for membrane attachment of a protein.[31] The membrane association of Tβγ which requires not only farnesylation but also methylation would facilitate the following reassociation of the dissociated partners on hydrolysis of GTP bound to Tα. It is still unclear whether or not the farnesyl/methyl-sensitive membrane binding of the dissociated Tβγ is mediated by a specific anchor protein in membranes, although the binding of doubly modified Tβγ (Tβγ-2b) was not affected at all by a limited proteolysis or a heat denaturation of rhodopsin, a major integral protein in photoreceptor membranes.[30] Rather, a recent study on the binding of Tβγ with artificial large unilamellar liposomes has pointed out the importance of the electrostatic interaction between Tβγ and the polar head groups of the lipid membranes.[30]

Taken together, the farnesylation and methylation at the C terminus of Tγ play dual roles. First, the modifications remarkably stabilize the α–βγ complex and consequently facilitate the coupling of trimeric transducin with metarhodopsin II, leading to acceleration of the GDP/GTP exchange reaction on Tα. Second, after activation of transducin, the modifications provide membrane anchoring of Tβγ which is dissociated from Tα–GTP. Investigation indicates that Tγ in bovine rod photoreceptors contains only the farnesylated and methylated form of Tβγ (Tβγ-2b) and suggests that Tγ-1 and Tγ-2a might be produced from Tβγ-2b during purification procedures owing to the chemical lability of the C-terminal structure of Tβγ-2b.[28] No experimental evidence has been obtained in our laboratory for the reversibility of the modifications,[28] though the methylation of Tγ was reported to be a reversible process.[32] These issues are to be elucidated in future studies aimed at providing a more detailed description of the function of lipid modifications on G-proteins.

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[10] Mutation and Analysis of Prenylation Signal Sequences

By ADRIENNE D. COX

Introduction

Proteins that are prenylated, or modified by the addition of isoprenoid lipids, direct this modification by means of consensus prenylation signal sequences at their carboxyl termini (reviewed in Refs. 1–3). The isoprenoid group(s) is attached via thioether bonds to one or more cysteine residues within the sequence, which is recognized by the prenyltransferase enzyme performing the attachment. Different classes of prenylation signals are recognized by different prenyltransferases, and some of the signal sequences also contain recognition motifs for other posttranslational modifications, including proteolytic cleavage and carboxylmethylation.[4,5] Proteins that are modified by prenylation include members of the ras superfamily of small GTPases, γ subunits of heterotrimeric G proteins, nuclear lamins, retinal signal transducing proteins, yeast transport proteins and mating pheromones, and viral proteins required for virion assembly.

It is sometimes desirable to mutate the prenylation motif of a protein in order to remove or alter the type of isoprenoid attached to it. Mutational analysis can complement genetic, pharmacological, and biochemical approaches to studying prenylation processes. For example, mutation of prenylation signal sequences to abolish prenylation of the encoded protein has demonstrated that the prenylation of proteins is critically important

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